Passage of cell-penetrating peptides across a human epithelial cell layer *in vitro*

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Cell barriers are essential for the maintenance and regulation of the microenvironments of the human body. Cell-penetrating peptides have simplified the delivery of bioactive cargoes across the plasma membrane. Here, the passage of three cell-penetrating peptides (transportan, the transportan analogue transportan 10, and penetratin) across a Caco-2 human colon cancer cell layer *in vitro* was investigated. The peptides were internalized into epithelial Caco-2 cells as visualized by indirect fluorescence microscopy and quantified by fluorimetry. Studies of peptide outflow from cells showed that the peptides were in equilibrium across the plasma membrane. The ability of the peptides to cross a Caco-2 cell layer was tested in a two-chambered model system. After 120 min, 7.0 %, 2.8 % and 0.6 % of added transportan, transportan 10 and penetratin respectively was detected in the lower chamber. Both transportan and transportan 10 reversibly decreased the trans-epithelial electrical resistance of the barrier model, with minimum values after 60 min of 46 % and 60 % of control respectively. Penetratin did not affect the resistance of the cell layer to the same extent. Although transportan markedly increased the passage of ions, the paracellular flux of 4.4 kDa fluorescein-labelled dextran was limited. In conclusion, the results indicate that the transportan peptides pass the epithelial cell layer mainly by a mechanism involving a transcellular pathway.

Key words: cell-penetrating peptide, drug delivery, epithelial barrier, penetratin, transcellular delivery, transportan.

INTRODUCTION

High-resistance epithelial/endothelial cell barriers restrict the passage of various hydrophilic compounds from the intestine and the circulating blood into the human body. The high resistance is due to the formation of well organized tight junctions that connect the cell plasma membranes via a network of apical localized seams. As the name implies, tight junctions exclude the paracellular passage of ions, peptides and proteins (for reviews, see [1,2]).

There are several cell culture models currently available for studying transport across tight junction barriers, and in the present study we have used the widely applied human epithelial Caco-2 cells. These cells in a two-chamber system are considered to be a model for predicting drug adsorption (reviewed in [3]).

Current techniques applied for the delivery of hydrophilic therapeutic agents over cellular barriers *in vivo* have several shortcomings, e.g. they are invasive and have a low yield of delivery [4,5]. CPPs (cell-penetrating peptides) [6], a novel type of cellular delivery agent, may prove to be a new and improved technique for delivery over a cell barrier. To address this possibility, we investigated whether CPPs would be as useful for delivery across a cell barrier as they have been proven to be for translocation across the cell plasma membrane.

Transportan is a 27-amino-acid chimaeric peptide containing the N-terminal 12 amino acids from the neuropeptide galanin and the entire sequence (14 amino acids) of wasp venom mastoparan (Table 1). A Lys^{13} residue was introduced to make labelling and cargo attachment convenient. Transportan has been shown to successfully deliver peptides, peptide nucleic acids and proteins into several different cell types [7,8]. The internalization of transportan is protein independent and, hence, is not mediated primarily through the endocytotic pathway [7].

A number of transportan analogues have been synthesized, and the most promising carrier candidate among these is TP10 (transportan 10), in which six of the N-terminal amino acids have been deleted [9] (Table 1). The well characterized CPP penetratin [10,11] was included throughout the study for comparison (Table 1). Transportan, TP10 and penetratin are representatives of two structurally divergent families of CPPs [12].

To our knowledge, this is the first study investigating the use of CPPs as potential carriers of therapeutics across an *in vitro* epithelial barrier. A recent study by Violini et al. [13] investigated the passage of a Tat fragment (residues 48–60) across Caco-2 cells. However, the ability of the Tat peptide to function as a true CPP is under debate [14].

To monitor that the tight junction barrier (the Caco-2 cell layer) remained intact, both the paracellular flux of dextran and the TEER (trans-epithelial electrical resistance) were measured. Additionally, the uptake and outflow of the peptides was characterized using the same cell line by both indirect and direct fluorescence detection. Peptide degradation was observed using MS.

MATERIALS AND METHODS

Peptide synthesis and labelling

The peptides (Table 1) were synthesized in a stepwise manner in a 0.1 mmol scale on a peptide synthesizer (model 431A; Applied Biosystems, Framingham, MA, U.S.A.) using a t-butoxycarbonyl strategy of solid-phase peptide synthesis.

Abbreviations used: Abz, 2-aminobenzoic acid; CPP, cell-penetrating peptide; HKR, Hepes-buffered Krebs–Ringer solution; log *P* value, logarithm of the octanol/buffer partition coefficient; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; TEER, trans-epithelial electrical resistance; TP10, transportan 10.

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Table 1 Sequences, molecular masses and log *P* values for the three CPPs examined

Molecular mass values are given with the fluorophore Abz attached. log *P* values were determined at a concentration of 10 μM Abz-labelled peptide in octanol and HKR buffer.

Name	Sequence	Molecular mass (Da)	log *P*
Transportan	GWTLNSAGYLLGK*INLKALAALAKKIL amide	2960	0.1 ± 0.1
TP10	- - - - - - AGYLLGK*INLKALAALAKKIL amide	2320	-0.5 ± 0.1
Penetratin	*RQIKIWFQNRRMKWKK amide	2360	-2.1 ± 0.1

* Labelled residue.

t-Butoxycarbonyl amino acids were coupled as hydroxybenzotriazole esters to a *p*-methylbenzylhydrylamine resin (Bachem, Bubendorf, Switzerland) to obtain C-terminally amidated peptides. Biotin or Abz (2-aminobenzoic acid) was coupled manually to a Lys ε-amino group for transportans or at the N-terminus for penetratin by adding a 3-fold excess of hydroxybenzotriazole- and *o*-benzotriazole-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluorborate-activated biotin or Abz (Chemicon, Stockholm, Sweden) in dimethylformamide to the peptidyl-resin after orthogonal deprotection from the Fmoc (fluoren-9-ylmethoxycarbonyl) group. The peptides were finally cleaved from the resin with liquid HF at 0 °C for 30 min in the presence of *p*-cresol. The purity of the peptides was > 98 %, as demonstrated by HPLC on an analytical Nucleosil 120–123 C-18 reverse-phase HPLC column (0.4 cm × 10 cm), and the correct molecular masses were obtained by using MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) MS (Voyager-DE STR; Applied Biosystems).

Cell culture and Transwell™ experiments

The human colon cancer cell line Caco-2 (A.T.C.C. via LGC, Borås, Sweden) was propagated in Dulbecco's modified essential medium with Glutamax (Invitrogen) supplemented with 10 % (v/v) foetal bovine serum, 1 mM sodium pyruvate, non-essential amino acids (1 × 100), 100 units/ml pencillin and 100 μg/ml streptomycin in air enriched with 5 % CO_2 at 37 °C.

Transwell® clear cups (0.4 μm pore size; Corning Costar) were coated with 0.5 μg/ml bovine plasma fibronectin (Invitrogen). A total of 100000 Caco-2 cells were seeded in each cup of a 12-well Transwell® (1.13 cm^2 filter area) and cultured for at least 10 days. The medium was changed in both the lower (1.5 ml) and the upper (0.5 ml) chamber every 2–3 days. Cell confluence was assessed using a phase-contrast microscope and by measuring the TEER with a Millicell-ERS (Millipore) with alternating current. As a control for cell layer permeability, the passage of FITC-labelled 4.4 kDa dextran (Sigma-Aldrich) was measured with or without EGTA treatment. Before the addition of peptides, the medium in the lower well was changed to HKR (Hepes-buffered Krebs–Ringer solution), in order to be able to detect the fluorescence of the Abz fluorophore. The resistance reached 800 $\Omega \cdot cm^2$ before the experiments were initiated; values of > 500 $\Omega \cdot cm^2$ are considered to represent high resistance [1].

Peptide at a concentration of 10 μM, dissolved in PBS, was added to the upper Transwell® chamber. At each time point, a 150 μl sample was collected from the lower chamber and replaced by fresh HKR. The fluorescence was measured at 320/420 nm (Abz) and 492/520nm (fluorescein) on a Spectramax Gemini XS (Molecular Devices). In addition, all experiments were performed at a peptide concentration of 20 μM; however, these data have been omitted from the graphs for clarity.

Peptide degradation studies in cells and in conditioned media using MALDI-TOF MS were performed as described previously [15].

As control experiments, the passage of peptides was performed with an identical set-up as for passage through the cell layer, but without the Caco-2 cells. This was carried out in order to quantify how much the Transwell® filter would limit the passage of the peptides.

Cellular penetration studies and fluorescence microscopy

The cells were grown on round glass coverslips (12 mm; GTF, Gothenburg, Sweden) in 24-well plates to approx. 50 % confluence. The medium was changed to serum-free medium and the biotinylated peptide solutions were added. The cells were incubated for 30 min at 37 °C or 4 °C. Cells were washed twice with PBS, fixed with 4 % (v/v) paraformaldehyde solution for 15 min at room temperature (in the dark) and then permeabilized in 30 mM Hepes buffer containing 0.5 % (w/v) Triton X-100 for 3 min on ice. Sites for unspecific binding were blocked in PBS containing 3 % (w/v) BSA overnight at 4 °C. Peptides were visualized by staining with avidin–FITC (Molecular Probes). The actin filaments were visualized with phalloidin/rhodamine (Molecular Probes) and occludin was stained with mouse anti-occludin antibody (Zymed, San Francisco, CA, U.S.A.) and a FITC-labelled anti-mouse antibody (Dakopatts, Täby, Sweden). The cell nuclei were stained with Hoechst 33258 (0.5 μg/ml) for 5 min, after which the coverslips were washed three times with PBS and mounted in 25 % (v/v) glycerol in PBS. The images were obtained with a Leica DM IRE2 fluorescence microscope and a Leica DC350 camera (Leica Microsystems) and processed with PhotoShop 5.5 software (Adobe Systems Inc.).

Peptide uptake and outflow studies in Caco-2 cells in suspension

Caco-2 cells were detached with trypsin (Invitrogen), resuspended in culture medium and centrifuged (1000 ***g*** for 10 min at room temperature). The cells were resuspended, counted and divided into aliquots in HKR on ice at 300000 cells/tube. Abz-labelled peptide (10 μM) was incubated for 15 and 30 min together with the cells in suspension in a total sample volume of 200 μl, on a shaking water bath at 37 °C. To stop uptake, trypsin solution was added for 3 min, in order to degrade the portion of peptide associated with the cell's plasma membrane. The cells were spun down at 1000 ***g*** for 10 min at 4 °C. The pellets were resuspended in HKR for fluorescence detection or, for the outflow samples, incubated again with peptide-free HKR. Fluorescence was read at 320/420 nm on a Spectramax Gemini XS (Molecular Devices). The intracellular concentration, or the amount of peptide protected from trypsin degradation, was calculated from a standard curve of Abz-labelled peptides. The average cell volume of Caco-2 cells was determined by using a Coulter 256 channelizer (Coulter Electronics Ltd).

Membrane disturbance assay

The efflux of radioactivity from cells preloaded with tritiated 2-deoxyglucose in the presence of peptide was measured according to a method described previously [16] with some modifications. Briefly, cells were seeded in 12-well plates and used for experiments 5 days after seeding, whereupon 0.5 μCi of 2-deoxy-D-[1–^{3}H]glucose (Amersham Pharmacia Biotech) in glucose-free

buffer was added to each well. The Caco-2 cells were preloaded for 20 min. Before sampling, the cells were washed rapidly with 3×1 ml of ice-cold PBS containing calcium and magnesium, to remove all extracellular radioactivity. Peptides in serum-free medium were added to the wells to final concentrations of 5, 10 and 20 μM. As a positive control, cells were treated with 1 % (v/v) Triton X-100 in PBS to establish the upper boundary of leakage. After 1, 5, 15 and 30 min, samples of 150 μl of medium were collected, Emulsifier Safe scintillation cocktail (Packard) was added and the radioactivity was measured in a Packard 3255 liquid scintillation counter. The remaining medium was collected from each well and placed into separate scintillation vials. The cells were dissolved in 0.5 ml of 1 M NaOH for 15 min. Cell fractions were placed in scintillation vials and the wells were rinsed with 0.5 ml of 1 M HCl, which was pooled with the NaOH fraction for neutralization. The relative radioactive efflux from each well was calculated as the percentage of untreated cells.

RESULTS

Fluorescence microscopy of cell penetration by CPPs in Caco-2 cells

Transportan, TP10 and penetratin entered Caco-2 cells at both 37 °C (Figure 1) and 4 °C (results not shown). Furthermore, addition of the endocytosis inhibitor phenylarsine oxide (25 μM) [17] did not affect the uptake of the peptides to any visible extent (Figures 1B, 1D, 1F and 1H). The transportan peptides were localized in the nuclear membrane and also in the nucleus, and yielded a diffuse staining throughout the cytoplasm. The staining of biotinylated penetratin differed: the nuclear staining was punctuate and the membrane was not stained to the same extent as for the transportan peptides.

The cellular uptake of CPPs in the present study was not temperature-dependent, which excludes uptake merely via endocytosis [18]. This is in agreement with earlier studies in which members of both peptide families, as well as other CPPs, have been shown to enter several different cell lines in a temperature-independent manner (for review, see [6]).

Quantification of cellular uptake and outflow

Outflow studies were performed to determine the ability of CPPs to exit cells. A trypsin wash of the cells was included to discriminate between cell-associated peptide (available for degradation) and internalized peptide (not available for degradation). After 15 min (results not shown), 30 % of the added transportan and 10 % of the added TP10 was found inside the cells; similar results were obtained at 30 min (Figure 2). Penetratin, on the other hand, showed 7 % uptake into Caco-2 cells in suspension after 15 min, which increased to almost 14 % after 30 min. From the number of cells and their average volume (0.98 pl per cell), the intracellular concentrations at equilibrium could be estimated to be 2 mM for transportan, 0.75 mM for TP10 and 0.95 mM for penetratin, at an extracellular peptide concentration of 10 μM (Figure 2). After 30 min in peptide-free HKR, the highest outflow was detected for penetratin (Table 2), of which only 18 % was retained in the cells, as compared with approx. 65 % for both transportans.

Effects of the CPPs on TEER and paracellular dextran flux

High resistance over an epithelial cell layer is due to the formation of well organized tight junctions, which prohibit the paracellular passage of proteins, peptides and ions [1]. Here we monitored both the TEER of the cell layer (Figure 3A) and the flux of fluorescent labelled dextran, which is of a comparable size to the peptides (Figure 3B). Taken together, the high TEER values and low flux of dextran confirm that the cell layer was intact when the treatments were started. The TEER of the barrier model was monitored from day 1 after seeding of the Caco-2 cells on fibronectin-coated Transwell® inserts, and normalized against inserts without cells. The experiments were initiated when the resistance had reached 800 $\Omega \cdot cm^2$, since values of over 500 $\Omega \cdot cm^2$ are considered to represent high resistance [1].

The passage of FITC-labelled dextran (4.4 kDa) increases when cells are treated with EGTA. The removal of calcium ions destroys the cadherin structures of the adherens junctions [19], leading to a break-up of the tight junctions. Calcium depletion has been used to establish the maximum flux via the paracellular pathway (Figures 3B and 4).

Both the TEER and dextran flux were measured after addition of the peptides to the confluent cell layer. At 10 μM, transportan and TP10 decreased the TEER to a minimum after 1 h of 46 % and 60 % respectively of control values (Figure 3A and Table 2). This is in contrast with the passage of dextran, where the flux was still limited (Figure 3B and Table 2). The effect of transportan was that only 0.5 % of added dextran was found in the lower chamber after 180 min (Table 2). The calcium-depleted cell layer had paracellular flux three times higher than that of the transportan-treated cell layer.

Passage of Abz-labelled peptides over cell layers

It has been found that 7–16 % of transportan translocates over the plasma membrane when added to various cell types [7,20]. However, a fraction of added peptide exits the cells [7], providing a mechanism for passage through the cell layer, but by a diffusion-like mechanism, using a transcellular pathway.

After 120 min, 7.0 % and 2.8 % of added transportan and TP10 respectively was found in the lower chamber (Table 2 and Figure 4). For comparison, only 0.6 % of added penetratin was found to cross the Transwell® cell layer. Passage was measured only from the apical to basolateral side of the Caco-2 cell layer. It has been shown previously that passive diffusion across an epithelial cell layer can occur in either direction [21]. The same percentage of peptide passed across the cell layer when the peptide concentration was increased from 10 μM to 20 μM in the upper Transwell® chamber, arguing in favour of transcellular equilibrium (results not shown)

Due to the effect of transportan on the TEER and the paracellular flux of dextran, the ability of peptides to cross the cell layer via the paracellular pathway was investigated further. For this purpose, biotinyl-transportan was added in combination with Abz-labelled penetratin. Surprisingly, only a minor effect on penetratin passage could be detected (from 0.6 ± 0.3 % to 1.0 ± 0.1 % of added penetratin; Figure 4).

Passage of Abz-labelled peptides over the Transwell® filter

To further investigate peptide passage across the cell layer in the Transwell®, peptide passage was performed without cells in the well. The Transwell® filter is made of polycarbonate with 0.4 μm pores, and the apical side of this filter was coated with bovine plasma fibronectin as described in the Materials and methods section. After 120 min, 1.4 % and 2.6 % of added transportan and TP10 respectively was found in the lower chamber (Table 2). For a comparison, 1.8 % of added penetratin was found to cross the filter of the Transwell®. Of the added reference compound,

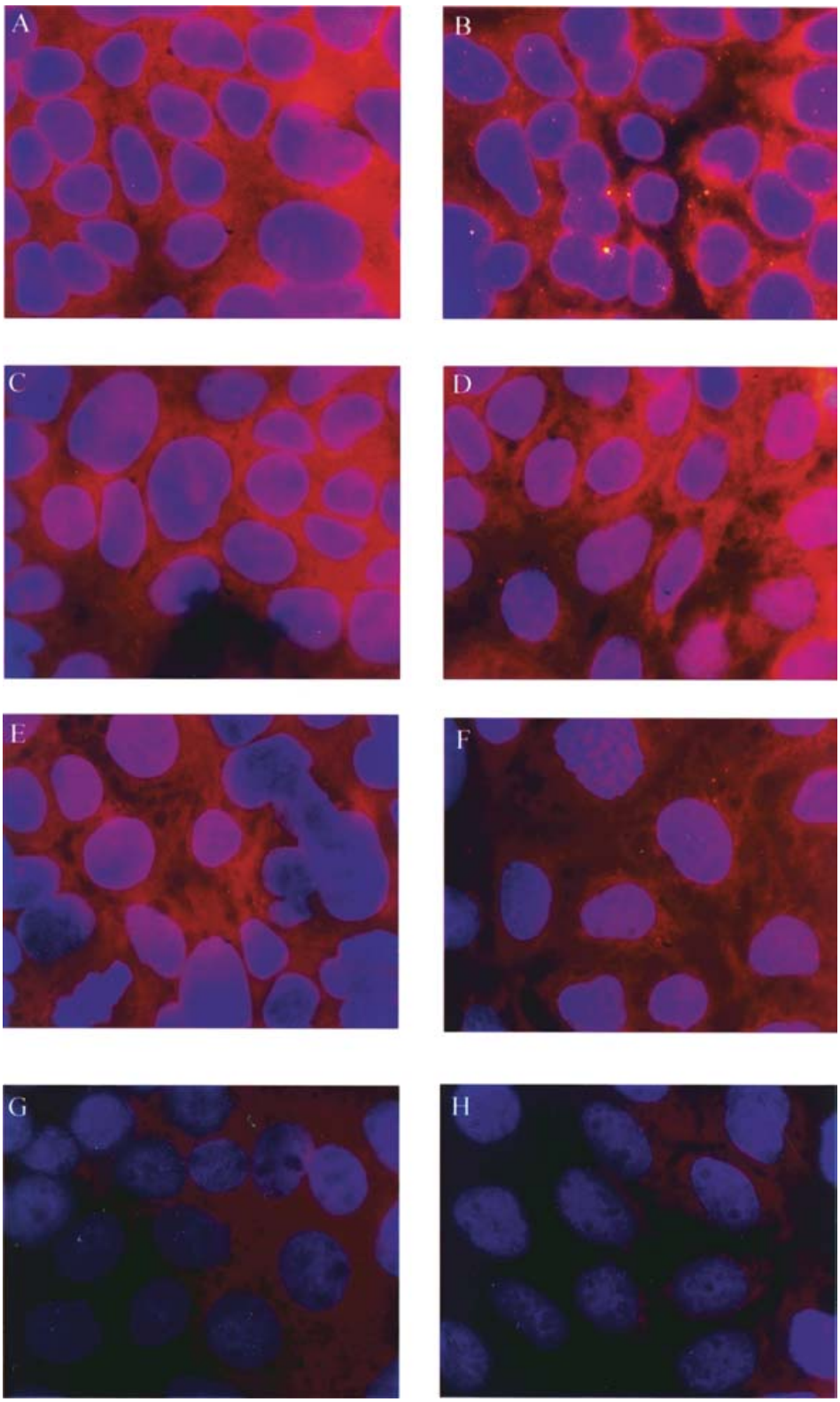

Figure 1 Internalization of biotinyl-peptides (10 μM) in confluent Caco-2 cells for 60 min at 37 °C

(**A**, **B**) Transportan, (**C**, **D**) TP10, (**E**, **F**) penetratin, and (**G**, **H**) without peptide. Peptides were visualized by indirect staining with avidin–TRITC (tetramethylrhodamine β-isothiocyanate) and nuclei were stained with Hoechst 33258. Panels (**B**), (**D**), (**F**) and (**H**) show cells treated with the endocytosis inhibitor phenylarsine oxide (25 μM).

FITC-dextran, 70 % was found in the lower chamber after 120 min (results not shown). Passage was measured in the same manner as described for passage through the cell layer.

To analyse peptide passage, the values from passage through the filter were treated as baseline values with regard to passage through the cell layer (Figure 5). Unexpectedly, transportan

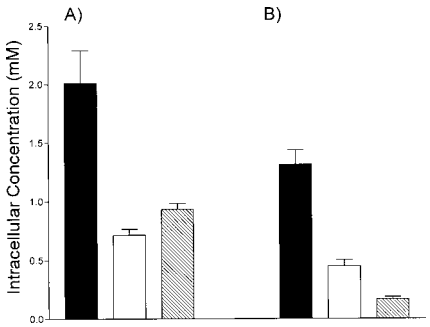

Figure 2 Retention of CPPs in Caco-2 cells in suspension

(**A**) Intracellular peptide concentration after a 30 min incubation with 10 μM Abz-labelled peptide in HKR buffer; (**B**) intracellular peptide concentration after incubation in peptide-free HKR buffer for 30 min. Transportan, TP10 and penetratin are indicated by solid, empty and hatched bars respectively. The error bars represent S.E.M. ($n \geqslant 3$).

passage was found to be five times greater through the cell layer than through the polycarbonate filter, TP10 displayed non-cell-dependent passage, whereas for penetratin the cell layer restricted passage.

Stability of the CPPs

The major peak in the mass spectra after 180 min in extracellular serum-free medium in contact with adhesive Caco-2 cells corresponds to the molecular mass of the intact peptide. Penetratin is rapidly degraded, probably due to its many dibasic cleavage sites. However, after 4 h of incubation, intact peptides were still detectable in cell lysates (results not shown), showing that the peptides were only partly degraded during this time. At least ten degradation products were found for both transportan and TP10. As demonstrated previously [9], the identified cleavage products would be inactive in cell penetration.

Due to the non-quantitative data from MALDI-TOF MS, only the relative half-lives of the peptides could be estimated in the cell lysate and extracellular medium. The stability of the peptides was in the order transportan > TP10 > penetratin. The main metabolite of the degraded transportan peptides appeared to be mastoparan-(3–14), as determined by MS analysis (mass 1430). Furthermore, qualitative MALDI-TOF analysis revealed the correct molecular mass of the intact peptides in the lower compartment after the Transwell® experiments (results not shown).

Effects of the CPPs on the plasma membrane

Several CPPs have been shown to be toxic, most likely due to disturbance of the plasma membrane at higher peptide concentrations [22]. To assess any possible membrane disturbance induced the peptides used in our experiments on Caco-2 cells, the glucose analogue 2-deoxyglucose, which is taken up into cells by glucose transporters and is phosphorylated inside the cells by hexokinase, was used. The product, 2-deoxy-D-[1-^{3}H]glucose 6-phosphate, cannot exit the cell via the plasma membrane [23]. Thus efflux of radioactivity from cells preloaded with ^{3}H-labelled 2-deoxyglucose requires some form of membrane disturbance.

After 30 min of treatment at a peptide concentration of 10 μM, there was no effect on the leakage of 2-deoxyglucose 6-phosphate;

Table 2 Toxicity, TEER, paracellular dextran flux, peptide passage and intracellular peptide retention for the three CPPs

Membrane disturbance is given as percentage retention of 2-deoxy-D-[1-^{3}H]glucose 6-phosphate radioactivity after incubation with the CPP for 30 min. TEER is given as % of control at 60 min, the time point of maximal effect (see Figure 3, left panel). Dextran flux is given as % of FITC–dextran added that has undergone paracellular flux in 180 min. Peptide flux was measured at 120 min. Intracellular retention of peptide is given as percentage retention of added peptide after 30 min outflow. Values are means ± S.E.M. ($n \geqslant 3$).

	Membrane disturbance (%)				Peptide passage (%)		
Peptide	10 μM	20 μM	TEER (%)	Dextran flux (%)	Cell layer	Filter	Intracellular retention of peptide (%)
Transportan	91.8 ± 1.6	71.0 ± 1.4	46.3 ± 2.5	0.46 ± 0.1	7.0 ± 0.6	1.4 ± 0.1	65 ± 2.9
TP10	93.7 ± 2.1	71.9 ± 2.1	60.1 ± 3.6	0.27 ± 0.1	2.8 ± 0.3	2.5 ± 0.7	63 ± 2.9
Penetratin	98.0 ± 2.1	87.5 ± 4.2	95.6 ± 2.6	0.20 ± 0.1	0.6 ± 0.3	1.8 ± 0.4	18 ± 1.1

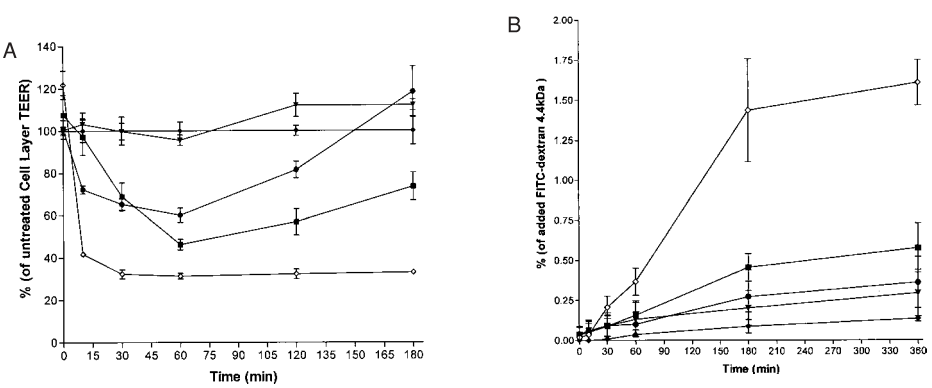

Figure 3 Permeability changes over time in CPP-treated Caco-2 cell layers

(**A**) TEER of the tight junction barrier model at different time points after addition of 10 μM peptide solution. (**B**) As a control for paracellular flux, FITC-labelled dextran of comparable size (4.4 kDa) was added together with the peptides. Values are given as a percentage of FITC–dextran added. Treatment with 10 mM EGTA was used to assess maximal paracellular flux (◇). ■, Transportan; ●, TP10; ▼, penetratin; ◆, untreated cell layer. Error bars indicate S.E.M. ($n \geqslant 3$).

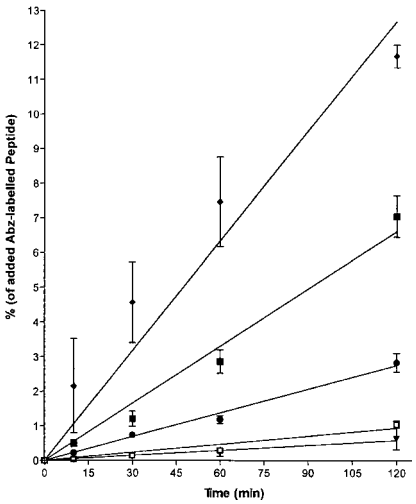

Figure 4 Passage of Abz-labelled CPPs over Caco-2 cell layers

■, Transportan; ●, TP10; ▼, penetratin; □, penetratin plus biotinyl-transportan. Values represent the percentage of the added peptide transferred to the lower compartment at different time points; error bars show S.E.M. ($n = 4$). The effect of treatment with 10 mM EGTA on the passage of Abz-transportan is also shown (◆).

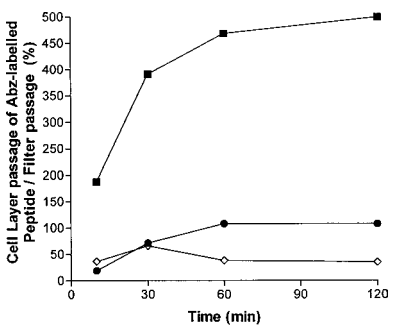

Figure 5 Passage of Abz-labelled CPPs across Caco-2 cell layers normalized to passage in the absence of cell layer in the Transwell® system

■, Transportan; ●, TP10; ◇, penetratin.

however, at 20 μM, both transportan- and TP10-treated cells retained only approx. 70 % of the total radioactivity. Penetratin caused lower leakage, with 87 % retention of radioactivity within cells (Table 2). This is in agreement with previous findings [15,24].

DISCUSSION

According to Lipinski's rules, drugs that are characterized by a log$P$ value (logarithm of the octanol/buffer partition coefficient) of between -1 and 5, have a molecular mass of approx. 500 Da, donate five or fewer hydrogen bonds and accept 10 or less hydrogen bonds are more likely to be absorbed from the intestine and "have better permeation" [25]. The CPPs in the present study deviate from Lipinski's rules, with regard to both molecular mass (2430–3070 Da) and the number of possible hydrogen bonds. However, CPPs have been demonstrated clearly to enter a vast variety of cell types, and in the present study we show that CPPs also have the potential to be used for delivery across a cell layer. Moreover, the log$P$ values of the CPPs seem to correlate with the percentages of the peptides that traverse the Caco-2 cell layer (Tables 1 and 2).

Transportan has been shown previously to be in equilibrium over the plasma membrane of Bowes cells [7]. Here we have shown that this is also true for Caco-2 cells (Figure 2). After a 30 min incubation in peptide-free buffer, approx. two-thirds of the transportan peptides was retained within the cells (i.e. not available for trypsin degradation). If the buffer was again changed at this point, a new equilibrium was established, where again two-thirds of the intracellular peptide was retained inside the cells (results not shown). For penetratin, on the other hand, only approx. one-fifth of added Abz-labelled peptide was retained in the cells. Scheller et al. [26] suggested that, due to the amphipathic character of several CPPs, such as transportan and TP10, they may interact with intracellular structures, which could explain their accumulation inside the cells. This would also clarify why the non-amphipathic penetratin is not retained inside cells, but rather 'washed out' [26]. In contradiction with this, however, only a low percentage (0.6 %) of added penetratin transited the polarized Caco-2 cell layer in the barrier model of the present study.

To further investigate penetratin's contradictory behaviour in outflow and passage through the cell layer, we studied peptide flow over fibronectin-coated Transwell® filters in the absence of cells and compared it with the peptides' passage over the cell layer. As expected, penetratin and dextran crossed the naked filter to a greater extent than the cell layer. However, the transportan peptides behaved differently; TP10 was found in comparable amounts in the lower well regardless of the presence of cells, while transportan crossed the cell layer in 5 times higher amounts with the cell barrier present (Figure 5). One could speculate that the cellular accumulation of transportan, seen in the outflow study, would lead to a large increase in peptide concentration close to the filter, which could aid the peptide to cross the polycarbonate filter. We had expected a correlation of peptide outflow and passage through the cell layer, but this was not the case, as penetratin had the highest rate of outflow (lowest retention), but showed the lowest percentage of added peptide found in the acceptor chamber.

If the low passage of the peptides across the Transwell® filter is interpreted as peptide binding to the coated filter, penetratin has a high affinity for the filter. This would also explain its low passage when co-added with transportan. However, transportan is also limited by the filter and was, in contradiction with penetratin, conveyed by the cells, possibly due to the marked intracellular accumulation (see Figure 5). Moreover, the efficiency of the passage of peptides correlates with their stability data. This may play a part in the low degree of penetratin passage, which could explain the biphasic behaviour apparent after 30 min in Figure 5. In addition, whether the cells where exposed to 10 or 20 μM peptide, the same percentage of added peptide was detected in the lower chamber (results not shown), which argues for an equilibrium-like behaviour of the peptides, not only over the plasma membrane but also over the cell layer.

Intestinal cells express peptide transporters that might increase uptake from the apical side. However, since these transporters typically import smaller peptides [21], and since the CPPs are imported at 4 °C, these transporters are probably not important in this process.

The increase in paracellular ion flux induced by transportans, as measured by TEER, was reversible. It reached a maximum after 1 h, was nearly reversed after 2 h (Figure 3B) and was fully reversed at 4.5 h (results not shown). Although transportan markedly increased ion passage (TEER), the paracellular flux of fluorescein-labelled 4.4 kDa dextran was still limited. This is in agreement with Ranaldi et al. [27], who also noted that at least a 50 % decrease in TEER was necessary for the increased passage

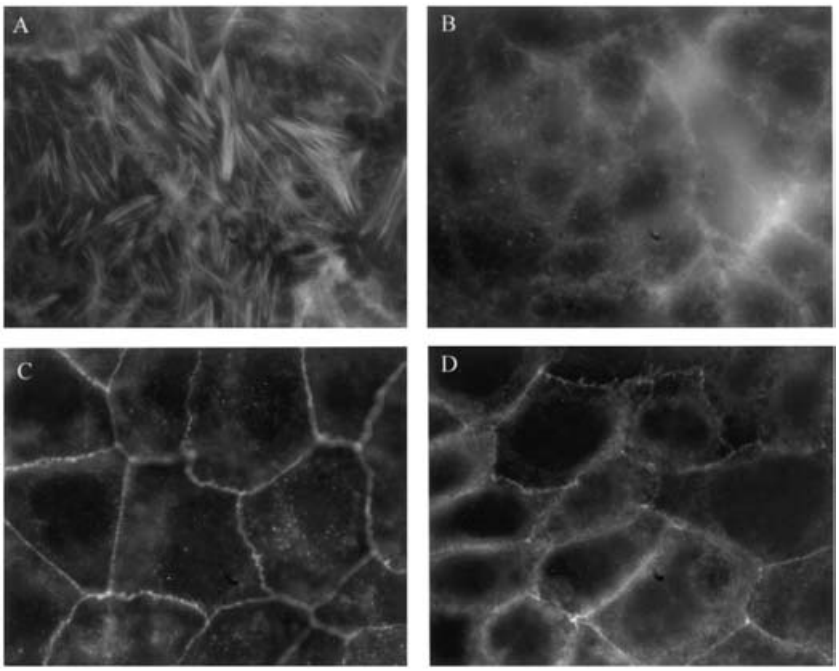

Figure 6 Visualization of the effects of transportan on actin filaments

(**A**, **C**) Confluent Caco-2 cell layers were treated with 10 μM transportan for 60 min at 37 °C; (**B**, **D**) untreated cells are shown for comparison. (**A**, **B**) Staining with rhodamine–phalloidin for visualization of actin filaments; (**C**, **D**) staining for the tight junction marker occludin.

of inulin (5000 Da). The increase in TEER indicates a break-up of tight junctions [3], whereas the adherens junctions may be intact, thus limiting the paracellular passage of larger molecules such as dextran and peptides. Yet, when cells treated with transportan for 1 h (which is the time point when the TEER was at its minimum) were stained for the tight junction marker occludin, no visible break-up of the junctions could be detected (Figures 6C and 6D). This implies that transportan treatment leaves the junctions more or less intact; hence the limited dextran flux [27].

Fluorescence staining with rhodamine-labelled phalloidin revealed marked reorganization of actin filaments when Caco-2 cells were treated with transportan peptides (Figure 6). The long vertical stress fibres were thicker and more pronounced as compared with those in untreated cells. Furthermore, actin reorganization was most distinct in the cells with the highest peptide staining. One could speculate that the close relationship between cell adhesion plaques and the actin cytoskeleton [28] may provide an explanation for the decrease in barrier resistance in cell layers treated with transportans. Penetratin, in comparison, did not reorganize the actin filaments, which were comparable with those in control cells at all time points (results not shown). Therefore the effect on actin is not an unspecific effect of CPPs, but is related to the biological activities of the transportans, such as the inactivation of ATPases [7] or GTPases [29].

Transportan peptides are known to disturb the plasma membrane in Bowes melanoma cells [24]; therefore, even though the effect on Caco-2 cells was small (Table 2), it is possible that the decrease in TEER reflects disturbance of the plasma membrane and not the break-up of tight junctions [1], as supported by the intact occludin staining (Figure 6). At higher CPP concentrations, dextran flux became more pronounced, especially when treated with transportan. However, the flux was still relatively low compared with that in EGTA-treated cell layers (Figure 3B). Consequently, we believe that transportan passes through the cell layer primarily via a transcellular pathway, at least at lower concentrations. Further evidence for the limited paracellular flux is that, in EGTA-treated cell layers, through which paracellular passage is unlimited, no great increase in transportan passage could be detected (Figure 4). Moreover, the uptake and outflow data demonstrate high uptake and outflow of these peptides in the same cells, making it probable that they pass the barrier via the transcellular route.

In summary, recent findings indicate that CPPs can be used to deliver therapeutics across a tight cell layer *in vivo* [30,31]. Here we show that transportan, TP10 and penetratin enter Caco-2 cells. Moreover, the transportan peptides are able to pass across a cell monolayer; penetratin can also do this, although in a lower yield, probably partially due to rapid degradation. The data presented in this study demonstrate that transportan and TP10 are conveyed over the epithelial cell layer mainly via the transcellular pathway; nevertheless, the paracellular pathway may contribute to the passage. The transportans also have a reversible effect on the permeability of the cell layer, which requires further investigation. This opens up the possibility that transportan and TP10 could be used as tools to deliver pharmacologically relevant substances across biological barriers.

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